A CHEMICAL AND PHYSICAL CHARACTERIZATION OF ECHINOID RNA DURING EARLY EMBRYOGENESIS

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ABSTRACT The properties of the major RNA components prepared from *Arbacia punctulata* have been characterized by sedimentation in sucrose gradients and analysis of base composition. Comparison of the components found in the unfertilized egg with those observed subsequent to fertilization revealed no differences in any of the embryonic stages examined. The base composition and sedimentation profiles of RNA from unfertilized nuclear egg fragments and from 105,000 *g* pellet were similar to those of the intact egg. It is concluded that the early stages of embryogenesis are not accompanied by detectable alteration of the physical or chemical characteristics of the major RNA components found in the unfertilized egg.

INTRODUCTION

Following fertilization or parthenogenic stimulation of the sea urchin egg, protein synthesis is initiated (Hultin, 1961). This event is not inhibited by the presence of actinomycin (Gross and Cousineau, 1963) or the absence of nuclear material (Denny and Tyler, 1964), nor is it paralleled by any chemically evident increment in net RNA synthesis (Schmidt et al., 1948; Villee et al., 1949). These observations suggest that the unfertilized echinoderm egg already possesses the genetic messages necessary for the initiation of embryogenesis. Subsequent development would, however, presumably be dependent on the transcription and translation of new information.

A satisfactory description of the relation between embryonic development and gene transcription will ultimately require: (*a*) identification of the particular messages responsible for individual events; and (*b*) an understanding of the mechanisms by which their translation is monitored and coordinated. Prerequisite to any experimental assessment of the former is a knowledge of the chemical and physical characteristics of the bulk RNA components present at different stages of development. With this information available, a meaningful search can be designed

for the relevant translatable fraction(s). It is the purpose of the present paper to provide some of the information required.

MATERIALS AND METHODS

Preparation of Eggs, Embryos, and Sperm

The sea urchin employed was *Arbacia punctulata,* obtained from Beaufort, North Carolina. Shedding of eggs and sperm was induced by the electrical shock technique of Harvey (1954). Unfertilized eggs were carefully examined for the presence of cytolyzed material, immature oöcytes, and such other contamination as is common to echinoid material, by the use of a Wild M5 stereomicroscope (Wild Heerbrugg Instruments Inc., Port Washington, New York). This enabled us to ascertain the biological integrity of large numbers of eggs per viewing field. Batches of eggs which did not appear to be at least 98% normal prior to fertilization, or that yielded less than a minimum of 95% normal development, were discarded. Clutches of eggs of from 1 to 4 females were combined subsequent to microscopic examination. Eggs were serially washed in approximately 1200 volumes of either filtered or artificial sea water (Tyler, 1953). Samples of material which were to be harvested in an unfertilized state were subjected to parallel developmental controls. Following fertilization, the zygotes were washed in an additional 300 ml of either filtered or artificial sea water. They were subsequently allowed to develop in large, shallow Pyrex dishes at the concentrations and depth necessary to prevent overcrowding and permit proper aeration. The temperature during development varied between 20 and 22°C. Synchronism of development was monitored by microscopic examination. Embryos were harvested and washed in an additional 500 volumes of sea water by sedimentation or (for swimming stages) by light centrifugation. Finally, the eggs and embryos were subjected to two washings via centrifugation. The resulting pellet was frozen in a dry ice methanol bath. The material was either stored at −18°C for 14 days and then transferred to a −70°C freezer, or stored directly at −70°C.

Preparation of Nucleated Egg Fragments

Arbacia punctulata unfertilized eggs were obtained and washed in the usual manner. All subsequent operations were carried out at 4°C. The gradient employed consisted of 5 ml each of the following sucrose solutions prepared in artificial sea water: 1.2 M (bottom layer), 7.5 M, 7.0 M, 6.0 M, and 5.5 M, (top layer) sucrose. Approximately 4 ml of a dense dejellied egg suspension was layered on top of the gradient. The eggs were subjected to centrifugation at 10,000 *g* and 4°C for 10 min in the SWB 25.1 rotor of the Spinco Model L preparative ultracentrifuge. Two distinct bands were noted following centrifugation. The upper band contained greater than 95% nucleated egg fragments, while the lower band consisted of a mixture of anucleated halves and intact cells. Most of the unbroken eggs found in the lower band exhibited a "dumbbell" appearance, and all were clearly stratified. The nucleated egg fragments were aspirated off, and pelleted by centrifugation at 6000 *g*. The pellet was stored at −70°C.

Preparation of RNA

60°C phenol extraction. The frozen pellet was homogenized at 4°C in solution I (0.001 M $MgCl_2$, 0.1 M NaCl, and 0.01 M K acetate, pH 5.2). The homogenate was made 1% with sodium dodecyl sulfate (SDS) and an equal volume of preheated, buffer-saturated phenol (hereinafter referred to as phenol) was added. This mixture was

vigorously shaken at 60°C for 3 min. The emulsion was rapidly chilled by immersion in a −20°C ice bath and centrifuged at 8000 *g* for 5 min. The initial aqueous phase and viscous interphase were re-extracted with an equal volume of phenol at 4°C for 15 min. This was followed by centrifugation at 8000 *g* for 5 min. The resulting aqueous phase was subsequently subjected to two additional 4°C phenol cycles. Following the final extraction, the aqueous phase was precipitated with two volumes of ethanol at −20°C. The precipitate was collected by centrifugation (8000 *g*, 5 min) and dissolved in from 1 to 2 ml of solution I. Residual phenol was removed by passing the solution through a 13 cm × 1.5 cm, G-25 coarse Sephadex column (Pharmacia Fine Chemicals, Inc., New Market, New Jersey). If the RNA was to be subjected to DNase treatment, the column was equilibrated with solution I at a pH of 7.0. Otherwise, the pH 5.2 buffer was employed. DNase (Worthington Biochemical Corporation, Freehold, New Jersey) was added to a final concentration of 10 μg/ml and the preparation was incubated at room temperature for 5 min with gentle mixing. The solution was then made 0.5% with respect to SDS and an equal volume of phenol was added. Deproteinization was accomplished by extraction at 4°C for 10 min, followed by centrifugation at 8000 *g* for 5 min. The aqueous phase was then subjected to three successive ethanol precipitations at −20°C. The final precipitate was dissolved in solution I and assayed for purity by virtue of its absorption spectrum between 220 and 290 mμ. DNase treatment was frequently omitted from the procedure if the RNA was obtained from stages preceding 5 hr blastula or if the material was not to be employed for base ratio analysis. In this case, the G-25 effluent was directly subjected to ethanol precipitation and assayed as described above.

4°C phenol extraction. Following homogenization in solution I, the initial 1% SDS-phenol extraction was carried out at room temperature for 5 min and subsequently continued at 4°C for an additional 15 min. The emulsion was centrifuged at 8000 *g* for 5 min. The supernatant was then processed as described for 60°C phenol extraction.

Ribosomal RNA. The frozen pellet was homogenized at 4°C in solution II (0.15 M sucrose, 0.1 M KCl, 0.01 M $MgCl_2$, and 0.05 M Tris, pH 7.8). The homogenate was centrifuged at 15,000 *g* for 10 min. The resulting supernatant was centrifuged at 105,000 *g* for 90 min in the No. 40 rotor of the Spinco Model L preparative ultracentrifuge. The supernatant was decanted and the pellet was rinsed twice with solution I. The pellet was suspended in this buffer and made 0.5% with SDS. The ribosomal RNA was deproteinized via four phenol cycles according to the 4°C phenol extraction protocol presented above.

105,000 g pellet RNA. Following homogenization in solution II, the total homogenate was centrifuged at 105,000 *g* in the No. 40 rotor of the Spinco Model L preparative ultracentrifuge. This supernatant was discarded and the pellet was washed with solution I. This pellet was significantly larger (approximately 3 times) than that obtained from ribosomal preparations prepared from similar quantities of material, and did not disperse as readily in solution I. The RNA associated with the pellet was subsequently purified according to the procedure presented for 4°C phenol extraction.

Nuclear merogon RNA. The RNA present in the nucleated egg fragments was prepared according to the procedures presented for 4°C phenol extraction.

Comments on the Isolation of RNA

Hot phenol extraction (60°C) allegedly yields a product which differs from that obtained by either room temperature or 4°C phenol procedures (Georgiev and Mantieva,

1962). In our hands, this procedure routinely increased the average yield of RNA. However, this increment appeared to be a function solely of the initial phenol extraction. Subsequent 60°C phenol extractions not only failed to increase the yield of RNA, but resulted in a progressive degradation which was dependent upon the number of hot phenol cycles performed. For this reason, hot phenol was employed only during the initial extraction.

DNase treatment was carried out only after the acetate buffer was adjusted to pH 7.0 and all phenol had been removed. The conditions reported by Glisin and Glisin (1964), in which the DNase treatment was performed at pH 5.0 on an aqueous phase containing residual phenol, were not considered optimal for enzymatic digestion of DNA.

With the exception of RNA extracted from nuclear egg fragments, most of our preparations were visibly contaminated with traces of echinochrome. Neither: (*a*) prolonged phenol extraction, (*b*) increased concentrations of SDS, (*c*) repetitive ethanol precipitations, (*d*) ether extraction, (*e*)chromatography on G-25 or G-200 Sephadex, nor (*f*) purification through sucrose density gradient centrifugation completely removes this pigment (Slater and Spiegelman, unpublished). The pigment is believed to be bound or associated with protein in vivo (Brachet, 1950). In contrast, the ineffectiveness of phenol or SDS in vitro might best be interpreted by assuming that the echinochrome is complexed with the RNA per se. Whether this is an artifact due to the extraction procedure, or a naturally occurring complex, has not been ascertained. The function of echinochrome is, to our knowledge, unknown. It should be emphasized, however, that all of the RNA utilized in this study possessed the spectral qualities (between 220 and 290 mμ) typical of pure RNA (Fig. 1). The preparations consistently exhibited minimal 260/280 mμ and 260/230 mμ extinction ratios of 2.0. Incubation with 10 μg of RNase (Sigma Chemical Company, St. Louis, Missouri, 5 × crystallized) at 30°C for 10 min, followed by sucrose density gradient sedimentation analysis revealed that absolutely no UV-absorbing RNase-resistant material was present. Although RNAs obtained from

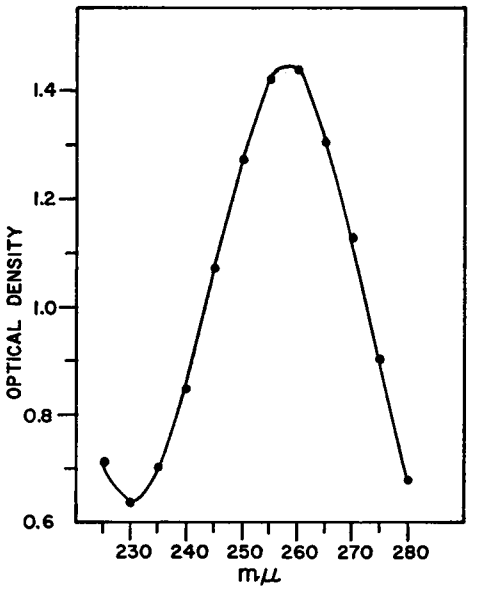


FIGURE 1 Absorption spectra of *Arbacia punctulata* unfertilized egg RNA.

Lytechinus variegatus and *Lytechinus pictus* eggs and embryos were free of any visible echinochrome contamination, a comparison of their sedimentation profiles with those of *Arbacia punctulata* failed to reveal any discernible differences (Slater and Spiegelman, unpublished). The elution profiles exhibited by the alkaline digests of *Arbacia* RNAs on Dowex (see section on *Base ratio analysis*) were qualitatively identical with those of *E. coli* RNA. No contaminating impurities could be optically detected regarding the 250/260 mμ or the 280/260 mμ ratios of either guanosine monophosphate or uridine monophosphate. The base composition of nuclear merogon RNA, which was ostensibly free of contamination, should also be noted (Table II). In view of the above, it is highly unlikely that trace quantities of echinochrome significantly influenced the results reported herein.

Preparation of DNA

Approximately 0.2 ml of sperm (frozen volume) was thawed in 10 ml of warm (50°C) SSC (0.15 M NaCl-0.015 M Na citrate, pH 7.0). Preincubated pronase (Calbiochem, Los Angeles, California) was added to a final concentration of 50 μg/ml and the solution was gently mixed for 30 min at 50°C. SDS was then added to a final concentration of 2.5%. Incubation was continued at 50°C for an additional 20 min. The now viscous suspension was diluted with 40 ml of room temperature EDTA saline (0.1 M ethylenediamine tetraacetate-SSC, pH 8.0) and subsequently made 1.0 M with sodium perchlorate. This mixture was briefly swirled at room temperature. An equal volume of chloroform-isoamyl alcohol (96:4 by volume) was added and the suspension was gently mixed at room temperature for 60 min. The emulsion was broken by centrifugation at 5000 *g* for 15 min. The aqueous layer was removed and subjected to three successive extractions with a water saturated phenol–chloroform-isoamyl solution (50:48:2). The phases were separated by centrifugation at 5000 *g* for 10 min. Following the final centrifugation, the DNA was precipitated from the aqueous layer by the dropwise addition of two volumes of cold (4°C) ethanol. The fibrous precipitate was spooled on a thin glass rod and dissolved in 10 ml of 1/10 SSC (0.015 M NaCl-0.0015 M Na citrate). The DNA was freed of any residual phenol and equilibrated with standard SSC by dialysis against SSC (100:1) for 8 hr with three changes of buffer.

Sucrose Density Gradient Centrifugation

Between 0.3 to 1.5 mg of RNA was layered over a 28 ml linear sucrose gradient (5 to 20%) prepared in solution I at pH 5.2. Centrifugation was for 10 hr at a prerun chamber setting of −10°C in the SWB 25.1 rotor of the Spinco Model L preparative ultracentrifuge. Following centrifugation, 1 ml samples were collected from the bottom of the tube and assayed for optical density at 260 mμ. Carrier herring sperm DNA (100 μg) was added to each tube and the samples were subsequently precipitated with 1 ml of cold (4°C) 20% TCA. The resulting precipitates were collected on coarse filter membranes (B6-27 mm of Schleicher and Schuell, Keene, New Hampshire) and extensively washed with cold 5% TCA. The filters were dried and subsequently counted in a liquid scintillation spectrometer (Packard Company, Des Plaines, Illinois).

Methylated Albumin-Kieselguhr Chromatography

Methylated albumin (MA) was prepared as previously described (Hayashi et al., 1965). Kieselguhr ("Hyflo-Supercel") was obtained from the Johns-Manville Products Corpora-

tion (Chicago, Illinois). A suspension of 4 gm of kieselguhr in 15 to 20 ml of Na-Po$_4$ buffer (1 part Na_2HPO_4 + 1 part NaH_2PO_4 to a final concentration of 0.05 M in PO_4 at pH 6.8) was heated to boiling and cooled to room temperature. One ml of 1% MA (prepared in distilled water) was added and the suspension was brought to 25 ml with phosphate buffer. Prior to use, the suspension was gently (to prevent denaturation) dispersed. A column 2 cm in diameter was constructed in the following manner. (*a*) The first layer (i.e. bottom layer) consisted of a 0.5 gm bed of powdered paper. (*b*) The second layer contained a mixture of 4 gm of kieselguhr (previously heated to boiling and cooled to room temperature in 20 ml of Na-PO_4 buffer) and 1 ml of 1% MA. (*c*) The third layer was prepared by mixing 3 gm of kieselguhr (previously heated to boiling and cooled to room temperature in 20 ml of Na-PO_4 buffer) with 5 ml of MAK. (*d*) The final (top) layer consisted of 0.5 gm of kieselguhr which had been heated and cooled to room temperature in 20 ml of Na-PO_4 buffer. All of the layers were individually washed down with Na-PO_4 buffer under 1 to 2 lb/in^2 of air pressure. The column was then washed with 100 ml of 0.45 M NaCl-Na-PO_4 buffer and loaded with 1 mg of RNA at a concentration of 50 μg/ml in this buffer under air pressure. The RNA was eluted with a 320 ml linear NaCl gradient containing from 0.50 M NaCl to 1.5 M NaCl in Na-PO_4 buffer, pH 6.8. Five ml fractions were collected and assayed for their optical density at 260 mμ. The NaCl concentration was monitored by measuring the refractive index of every fifth fraction.

Optical Density Temperature Profiles

The DNA or RNA to be melted was equilibrated by dialysis against SSC at 4°C. The nucleic acid solutions were adjusted to contain approximately 20 μg/ml in SSC and placed in covered quartz cuvettes. The cuvettes were positioned in a wired block (to permit controlled electrical heating) which fits the sample chamber of an Optica spectrophotometer (National Instrument Laboratories, Inc., Washington, D. C.). The temperature was monitored by a reference cell containing a thermistor. No corrections were made for cell or liquid expansion.

Base Ratio Analysis

The method employed was basically that of Katz and Comb (1963). Between 100 to 200 μg of RNA of known physical integrity (as judged by sucrose density gradient centrifugation) was hydrolyzed with 0.3 M KOH in a total volume not exceeding 0.5 ml for 18 hr at 37°C. The hydrolysis products were neutralized with cold (4°C) $HClO_4$ and the resulting precipitate removed by centrifugation. The supernatant was then made 0.05 N with respect to HCl. This sample was loaded on a 0.9 cm × 5.0 cm column containing Dowex 50-H^+, 200 to 400 mesh, 4× crosslinked (J. T. Baker Chemical Company, Phillipsburg, New Jersey). The column had been extensively washed with 3 N HCl and neutralized with H_2O. Prior to loading, it was equilibrated with 20 ml of 0.05 N HCl. The RNA digest was washed into the resin with 1 ml of 0.05 N HCl. Uridine monophosphate (UMP) was eluted first by the addition of 5 ml of 0.05 N HCl. The remaining nucleotides were eluted with a total of 40 ml of H_2O. Guanosine monophosphate (GMP) comes off as a single sharp peak (as does UMP). This is followed by a more diffuse peak containing adenosine monophosphate (AMP) and cytidine monophosphate (CMP). One milliliter samples were collected by allowing the effluent to drip directly into the volumetric funnel of an automatic fraction collector. The fractions

containing GMP, AMP, and CMP were made 0.05 N with respect to HCl before being assayed for their optical density. The respective absorbance of AMP and CMP was calculated according to the relationship of Loring (1955). The following millimolar extinction coefficients and wavelengths were employed: UMP, 10.0 at 260 mμ; GMP, 11.8 at 260 mμ; AMP, 14.4 at 257 mμ; and CMP, 13.0 at 279 mμ. A comparison of the base ratios of *E. coli* bulk RNA determined by means of chromatography on Dowex-formate columns (via ultraviolet absorbancy) by Midgley (1962) and by Hayashi and Spiegelman (1961) with those we have obtained employing Dowex 50-H^+ is presented in Table I.

TABLE I

BASE COMPOSITION OF *E. COLI* BULK RNA IN MOLES PER CENT

	Midgley	Hayashi and Spiegelman	This paper
moles %			
UMP	20.5	19.7	21.0
GMP	32.4	31.0	32.2
AMP	25.1	25.0	23.9
CMP	22.0	24.3	23.0

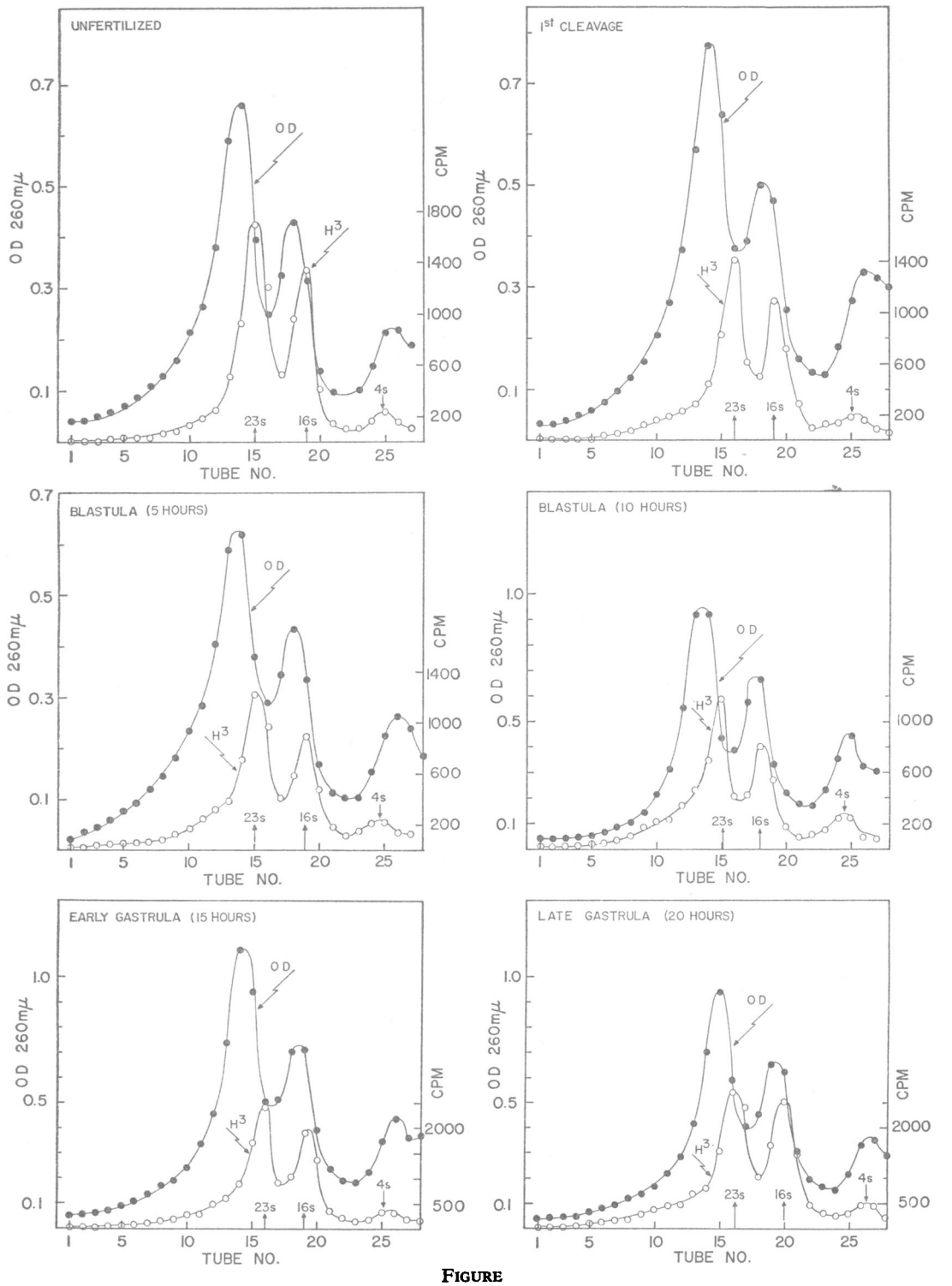
CsCl Density Equilibrium Centrifugation

The density of *Arbacia punctulata* sperm DNA was determined by centrifugation in a Spinco Model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California) using ultraviolet optics. Sperm DNA (2 μg) was centrifuged in CsCl with N^{15}-*Pseudomonas aeruginosa* DNA (1 μg) at 44,700 RPM and 25°C for 24 hr. Ultraviolet absorption photography of equilibrated DNA were traced on a microdensitometer (Joyce-Loebl double beam automatic recording microdensitometer, National Instrument Laboratories, Inc., Washington, D. C.). The N^{15}-*pseudomonas aeruginosa* DNA (density = 1.742 g/cm^3) provided a reference marker from which the density of the sperm DNA was determined. The method of Sueoka (1961) was employed to calculate the density and correlated GC content.

RESULTS

Sedimentation Profiles of Total and Ribosomal RNA as a Function of Development through Gastrulation. The total ethanol precipitable RNA (total RNA) present at various stages of development up to and including gastrulation was characterized by sucrose density gradient centrifugation (Fig. 2). The inclusion of a reference marker in the form of H^3 *B. megaterium* or *E. coli* RNA permitted a direct comparison of the sedimentation properties of all RNAs examined. It also served to preclude confusion with any artifactual differences which can readily arise from minor gradient disturbances.

The *Arbacia* RNA employed in this study was prepared via hot (60°C) phenol extraction as described in the Methods section. This procedure yields a product which exhibits a different sedimentation pattern from that obtained with cold (4°C)


UNFERTILIZED
1st CLEAVAGE
BLASTULA (5 HOURS)
BLASTULA (10 HOURS)
EARLY GASTRULA (15 HOURS)
LATE GASTRULA (20 HOURS)
O D 260mμ
CPM
TUBE NO.
O D
H3
23s
16s
4s

FIGURE

or room temperature phenol extraction (Fig. 3) resulting in a disproportionate increase in lower molecular weight RNA and a limited but reproducible increment in heavier material. A consistent shift in the position of the 16S to 18S peak relative to the 16S marker was noted, and appeared to be contingent upon the procedure employed to prepare the RNA. The over-all general increase in heterogeneity resulted in a loss of resolution between major RNA components. Limited degrada-

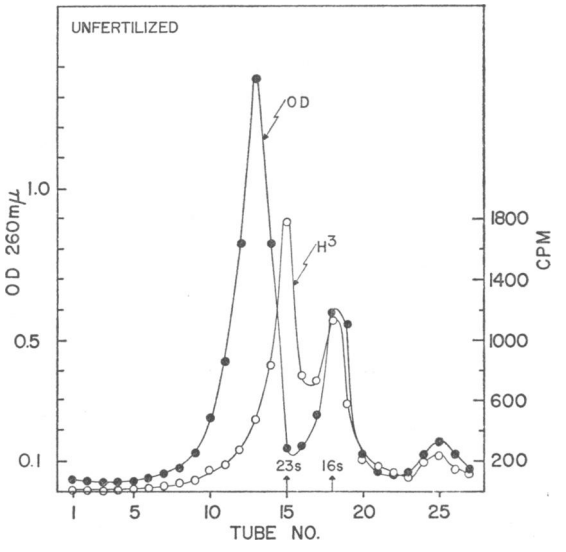


FIGURE 3 Sedimentation analysis of *Arbacia punctulata* total RNA prepared for eggs via 4°C phenol extraction. Unlabeled *Arbacia* RNA (336 μg) was mixed with H^3 *B megaterium* RNA (0.1 μg) and subjected to conditions of centrifugation and assay identical with those described in Fig. 2.

tion (which is not mitigated by the presence of bentonite) and artificial aggregation may be the basis for this alteration in sedimentation properties. However, in accord with similar results obtained in other laboratories (Rake and Graham, 1964; McCarthy and Hoyer, 1964; Cartouzou et al., 1965), the use of hot phenol did serve to significantly increase the average yield of RNA. It was therefore employed for the sedimentation studies presented in Fig. 1 to minimize sampling errors and

FIGURE 2 Sedimentation profiles of *Arbacia punctulata* total RNA from eggs and embryos at different developmental stages. All embryonic RNA employed in Fig. 1 was prepared via 60°C phenol extraction as described in Materials and Methods. *B. megaterium* H^3-RNA was included as a reference marker to permit direct comparison of the sedimentation properties of RNAs obtained from different stages of development. Between 300 and 450 μg of unlabeled *Arbacia* RNA was mixed with 0.1 μg of H^3 *B. megaterium* RNA and sedimentated through a 5 to 20% sucrose gradient (pH 5.2) at 25,000 RPM for 10 hr. The prerun chamber temperature was −10°C. One milliliter aliquotes were collected from the bottom of the tube (i.e. fraction No. 1) and assayed for their optical density at 260 mμ. The samples were subsequently precipitated with an equal volume of 20% TCA and counted on membrane filters in a liquid scintillation spectrometer as detailed in the Materials and Methods section.

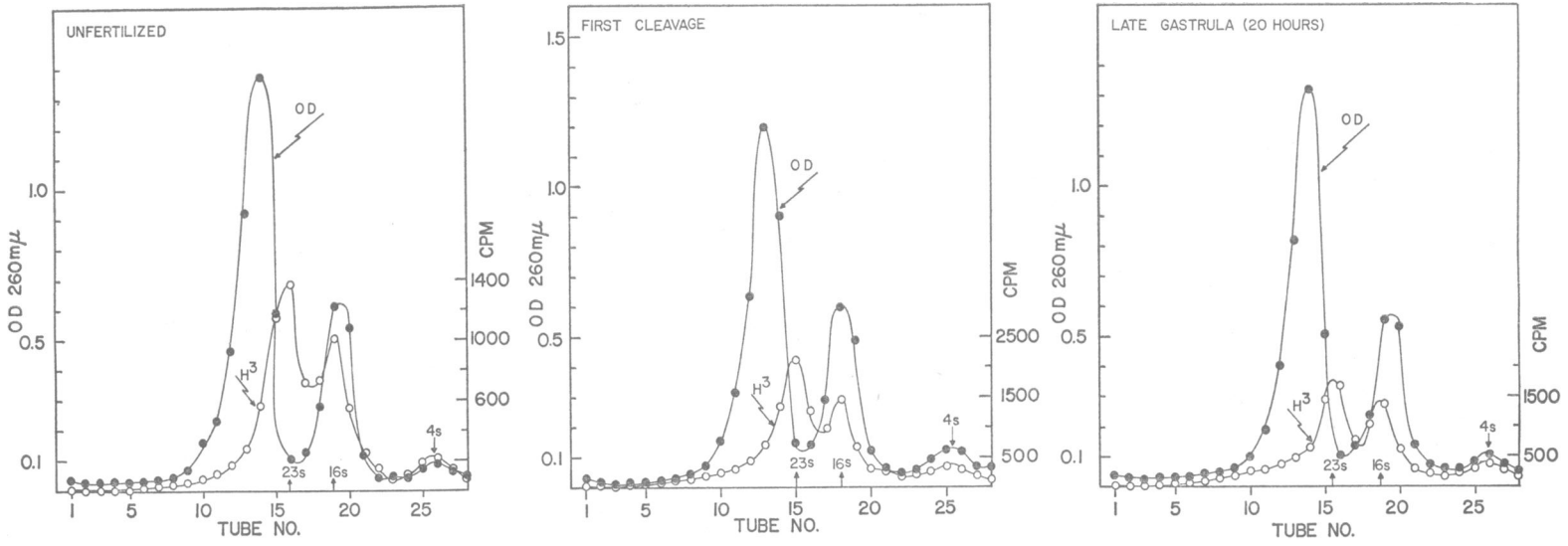


FIGURE 4 Sedimentation analysis of *Arbacia punctulata* ribosomal RNA. Ribosomal RNAs were prepared from unfertilized eggs, first cleavage material, and 20 hr gastrula. Each profile represents from 335 μg to 344 μg of embryonic RNA and 0.1 μg of H^3 *B. megaterium* RNA. The conditions of centrifugation and assay are indicated in Fig. 2.

thus obtain a representative spectrum of the RNA components present during development.

The profiles exhibited a marked degree of similarity. No additions or deletions to major components present prior to fertilization were encountered in any of the stages examined. The ratio of the 28S to 18S RNA displayed only minor fluctuations. A somewhat greater variation was observed in the proportion of soluble RNA present from first cleavage through late blastula. No UV absorbing RNase resistant material was detectable in the RNA preparation from unfertilized eggs.

The increase in amino acid incorporation that follows fertilization is reportedly related to ribosomal competence (Hultin, 1961; Monroy et al., 1965). A comparison (Fig. 4) of the RNAs derived from the latent ribosomes existing in unfertilized eggs and their metabolically active counterparts present in first cleavage and gastrula material failed to reflect this transition. This is even more forcefully demonstrated by the superimposed sedimentation profiles of unfertilized and gastrula ribosomal RNA presented in Fig. 5. Thus, absolutely no alteration in the physical properties of the ribosomal associated RNA as a function of fertilization and subsequent development could be detected by density gradient centrifugation.

A substantial decrease in the net yield of ribosomal RNA was observed following fertilization and first cleavage. Microscopic examination ruled out the possibility that this was due to incomplete cell breakage. The loss may be tentatively attributed

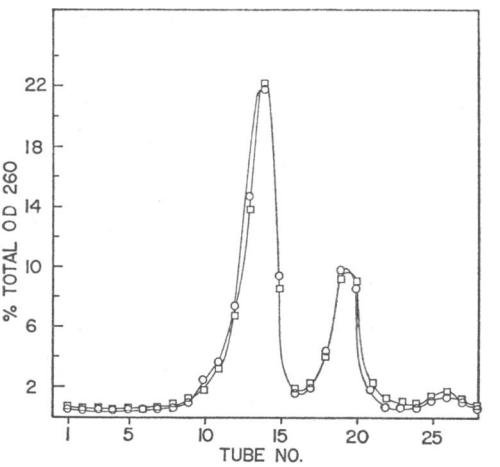


FIGURE 5 Comparative sedimentation profiles of *Arbacia punctulata* unfertilized egg and gastrula ribosomal RNA. Ribosomal RNA obtained from unfertilized eggs and 20 hr gastrula was subjected to cocentrifugation in separate swinging buckets under the conditions described in Fig. 2. The profiles are plotted according to the percentage per tube of the total optical density units employed per run (i.e. per cent total optical density). This permits a direct comparison of unfertilized eggs and gastrula ribosomal RNAs. A total of 6.3 and 6.0 optical density units were employed for unfertilized egg and gastrula ribosomal RNA respectively. Unfertilized egg ribosomal RNA is represented by circles and gastrula ribosomal RNA, by squares.

to either a complexing of ribosomal material with the membrane fraction pelleted in the initial centrifugation, or to progressive ribosomal degradation as a function of development (Comb and Brown, 1964).

Base Composition of Total and Ribosomal RNA Obtained from Unfertilized Eggs and Gastrula. The initial phase of active protein metabolism which takes place following fertilization reportedly reaches its maximum at the 32 to 64 cell stage. Its subsequent gradual decline through early blastulation is believed to be terminated in a new burst of activity during gastrulation (Giudice et al., 1962). As indicated above, no concomitant change in the physical properties of the RNA present during this period was noted. Characterization of the base composition of unfertilized and gastrula phenol-extracted RNA possessing known physical properties permitted a chemical comparison of the RNA existing at these two metabolically diverse stages.

The base composition of RNAs obtained from unfertilized eggs and gastrula repeatedly displayed a pronounced similarity (Table II). A greater variation in the percentage of UMP and GMP was observed to occur between ribosomal as-

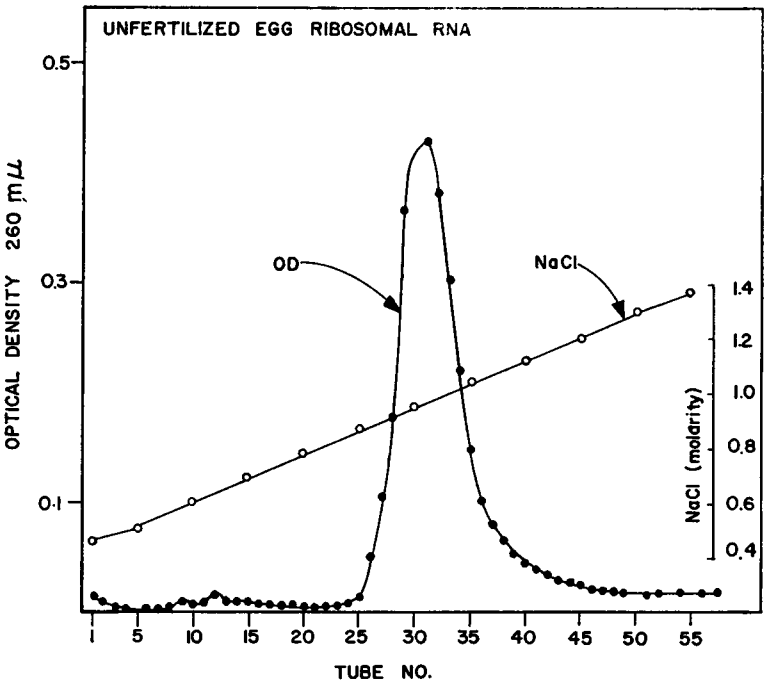


FIGURE 6 Methylated albumin-kieselguhr (MAK) column chromatography of *Arbacia punctulata* unfertilized egg ribosomal RNA. The MAK column was prepared as described in Materials and Methods. Approximately 1.0 mg of ribosomal RNA was loaded at 50 μg per ml in 0.50 M NaCl-0.05 M $Na\text{-}PO_4$ and eluted with a 320 ml linear gradient running from 0.45 M to 1.5 M NaCl in 0.05 M $Na\text{-}PO_4$, pH 6.8. Each fraction contained 5 ml.

sociated RNA and total cellular RNA (Table II). The contribution of 4S material to this consistent, but not of necessity significant, difference was examined in unfertilized eggs. Total RNA was prepared by the hot phenol procedure and via 4°C phenol extraction. Subsequent density gradient centrifugation and concentration yielded products free of soluble RNA. Base ratio analysis did not reveal any difference between ribosomal and total RNAs devoid of 4S material, with the possible exception of a minor fluctuation in GMP. Complete correspondence between the 60°C and 4°C products was also noted (Table II). These results indicate that the metabolic modulations prerequisite to gastrulation are not accompanied by any chemically detectable changes in the ribonucleotide composition of the high molecular weight RNA.

Chromatography on methylated albumin columns (Fig. 6) failed to resolve ribosomal RNA into the 28S and 18S components routinely obtained by density gradient centrifugation. That the pattern of elution from such columns is contingent upon both molecular weight and base composition (Spiegelman, 1963) suggested that the 28S moiety might possess a higher GC content (which elutes at a lower salt molarity) than that of its 18S counterpart, and thus compensate for the discrepancy in molecular weight. Optical density temperature profiles (Fig. 7) and base ratio

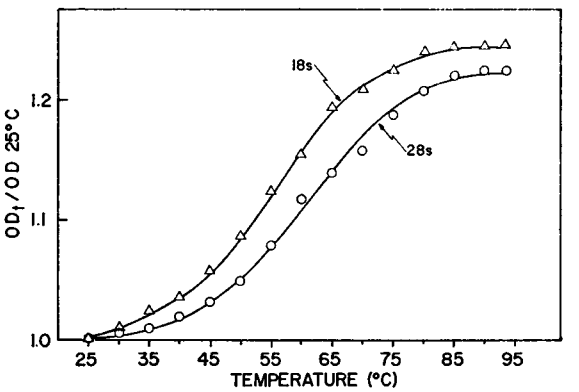


FIGURE 7 Optical density-temperature profiles of 28S and 18S *Arbacia punctulata* ribosomal RNA. The two major ribosomal RNA components were separated by density gradient centrifugation (Fig. 8) and subsequently equilibrated with 0.15 M NaCl-0.015 M Na citrate (SSC). Concomitant melting profiles were performed in SSC. *E. coli* DNA (not shown) served as a control. Both the 28S and 18S melting curves were almost completely reversible upon cooling, thereby indicating that the bonds involved are of an intramolecular nature. The hyperchromic shift is plotted in terms of the ratio of the optical density at 260 mμ at ambient temperatures (OD) to that at 25°C. The mean thermal stability of RNA is believed to be dependent on its GC content (Spirin, 1964). Although the gradual transition in melting did not permit a precise determination of the respective melting temperatures, the approximate Tm of the 28S RNA (61°C) proved to be 5° higher than that of its 18S counterpart (56°C). This suggested that the 28S RNA might possess a somewhat greater GC content than that of the 18S component, as was subsequently confirmed by base ratio analysis (Table II).

analysis (Table II) of purified 28S and 18S RNA (Fig. 8) strengthens this interpretation. Differences in the base composition of metazoan 28S and 18S RNA have been reported by at least four other laboratories employing some six different types of material (Montagnier and Bellamy, 1964; Harel et al., 1963; Brown and Gurdon, 1964; Brawerman et al., 1965). In all cases the 28S ribosomal RNA consistently exhibited a higher GC content than that of the 18S component.

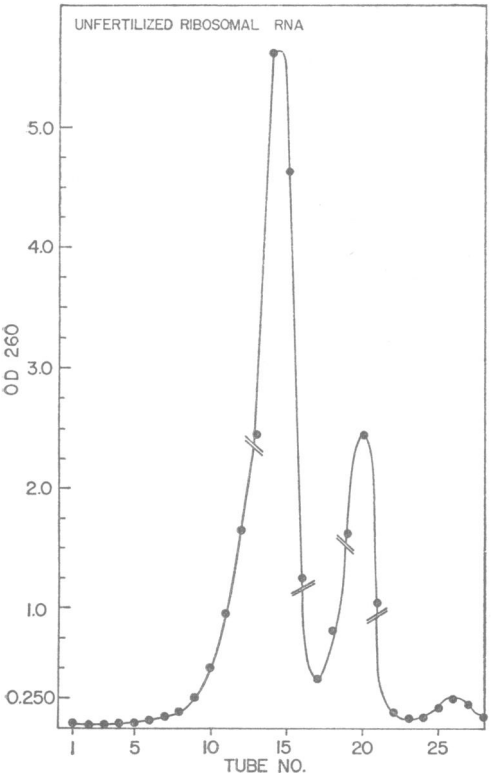


FIGURE 8 Preparative sedimentation profile of *Arbacia punctulata* unfertilized egg ribosomal RNA. Ribosomal RNA (1.3 mg) prepared as described in Materials and Methods, was subjected to density gradient centrifugation as detailed in Fig. 2. Fractions corresponding to the 28S and 18S components respectively, were combined and concentrated by ethanol precipitation. This material was subsequently employed for optical density temperature profiles (Fig. 7) and base ratio analysis (Table II) of 28S and 18S ribosomal RNA.

Melting point analysis and CsCl density equilibrium centrifugation were performed on *Arbacia* sperm DNA to determine whether any of the species of RNA examined possessed a base composition similar to that of *Arbacia* DNA. Thermal denaturation resulted in a 41% increment in optical density (Fig. 9), thereby indicating that the DNA samples employed were predominantly in a native configuration. The Tm of 84.5°C obtained from this monophasic melting curve corresponded to a value of 37% GC (Marmur and Doty, 1962). Centrifugation in CsCl established that the DNA was of high molecular weight by virtue of the narrow width of the band, and possessed no satellite component (Fig. 10). The density and correlated GC content were calculated according to the method of Sueoka (1961).

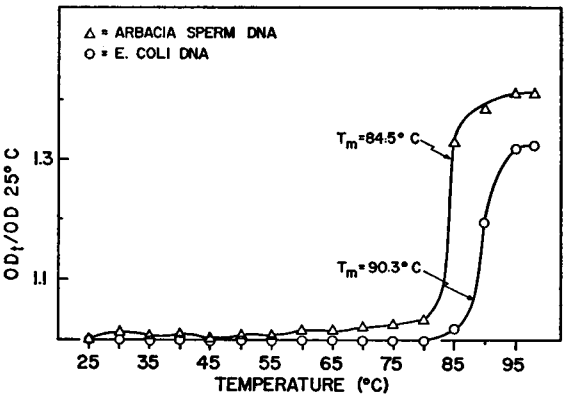


FIGURE 9 Optical density temperature profiles of *Arbacia punctulata* sperm and *E. coli* DNA. *Arbacia* sperm DNA was prepared as described in Materials and Methods. *E. coli* DNA was employed as a control. Both DNAs were equilibrated with SSC prior to melting. The *Arbacia* Tm of 84.5°C corresponded to a value of 37% GC (Marmur and Doty, 1962). Thermal denaturation resulted in a 41% increment in the optical density of the sperm DNA. The melting curve is plotted as described in Fig. 7.

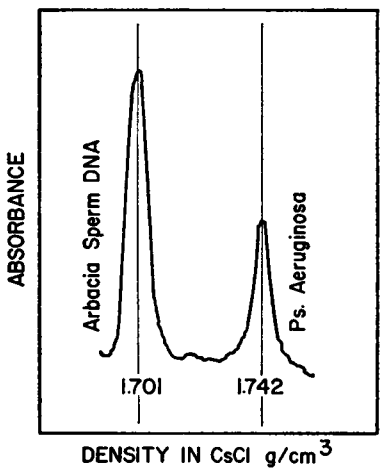


FIGURE 10 CsCl density equilibrium centrifugation tracing of *Arbacia punctulata* sperm DNA. *Arbacia* sperm DNA (2 μg) was centrifuged in CsCl with N^{15}-*Pseudomonas aeruginosa* DNA (1 μg) at 44,770 RPM and 25°C for 24 hr. Ultraviolet absorption photographs of equilibrated DNA were traced on a microdensitometer. The N^{15}-*Pseudomonas aeruginosa* DNA (density = 1.742 g/cm^3) provided a reference marker from which the density of the sperm DNA was determined. The method of Sueoka (1961) was employed to calculate the density and correlated GC content. Using this relationship, the derived density of 1.701 converts to 37.9% GC.

Using this relationship, the derived density of 1.701 converts to a per cent GC of 37.9. Carden et al. (1965), employing the Schildkraut et al. (1962) relationship have reported a GC content of 41% for *Arbacia* sperm DNA. This 3% discrepancy may be due to the different formulas employed [e.g. the reported per cent GC of mouse spleen DNA is 42% according to Sueoka (1961), and 44% according to Schildkraut et al. (1962)]. The 37.9% GC value does, however, appear to be in better agreement with the above mentioned melting point data and with a previously published chemical determination (Daly et al., 1950). It is evident from Table II

that regardless which figure is employed, the base composition of the various RNAs consistently failed to mimic that of the DNA.

The Distribution of RNA within the Unfertilized Egg.

Characterization of RNA associated with the 105,000 g pellet. The egg's cortex and cytoplasmic particulate inclusions have been classically considered as potential carriers of the developmental code (Raven, 1961). In view of the various roles assigned to these entities, an attempt was made to characterize the RNA associated with this material in the absence of the free or nonsedimental RNA present in total cellular extracts. This was accomplished by subjecting the total homogenate to a 90 min, 105,000 *g* centrifugation. The resulting pellet therefore contained not only ribosomes, but the bulk of the egg's cortical gel, membrane systems, and cell organelles. The RNA from this pellet was purified by 4°C phenol extraction, and characterized via sucrose density gradient centrifugation and base ratio analysis. The sedimentation profile obtained resembled that of ribosomal RNA with the exception of a slight increment in 4S material. Base ratio analysis revealed only minor deviations in the guanine and uridine content (Table II).

Characterization of RNA associated with nucleated egg fragments. Unfertilized eggs can be broken into two halves of approximately equal size by density gradient centrifugation (Harvey, 1932). Although both halves respond to parthenogenic stimulation or fertilization, their potentials of subsequent differentiation differ. The nucleated halves have been observed to form well developed plutei. Cleavage in parthenogenically activated anucleate merogons normally terminates

TABLE II

COMPARATIVE BASE RATIOS OF *ARBACIA PUNCTULATA* EGG AND GASTRULA RNAS

	UMP	GMP	AMP	CMP	% GC	Pu/Pyr
			mole %			
Unfertilized:						
Total*	24.4	30.8	20.6	24.2	55.0	1.05
Total minus soluble RNA*	21.8	32.1	20.7	25.5	57.6	1.11
Total minus soluble RNA‡	22.1	31.9	20.6	25.4	57.3	1.10
105,000 *g* pellet RNA‡	22.8	31.6	21.1	24.6	56.2	1.12
Nuclear merogon total RNA‡	23.3	31.3	20.2	25.2	56.5	1.18
Ribosmal	21.9	34.1	19.8	24.2	58.3	1.29
18S	23.2	29.9	23.1	23.8	53.7	1.12
28S	19.3	34.9	19.9	26.0	60.9	1.20
Gastrula:						
Total*	24.1	32.1	20.5	23.4	55.5	1.10
Ribosomal	20.6	32.4	21.7	25.3	57.7	1.27

* 60°C phenol extraction.
‡ 4°C phenol extraction.

in poorly defined blastulae or abnormal cytoplasmic masses (Harvey, 1940). Following fertilization or parthenogenic stimulation, however, both egg fragments display a similar activation of protein synthesis as witnessed by amino acid incorporation (Denny and Tyler, 1964; Baltus et al., 1965; Malkin, 1954). To determine whether a discernible qualitative polarity exists in the unfertilized egg with respect to RNA, sucrose density gradient profiles and base composition analysis were performed on nucleated egg fragments.

Total RNA obtained from nucleated egg fragments exhibited a somewhat more discrete composition with respect to the spectrum of banding of 28S and 18S components, than that commonly obtained with total RNA derived from the whole egg (Figs. 3 and 11). The experimental limits of the techniques employed, however, do not permit the assumption that this tenuous difference is real. Base ratio

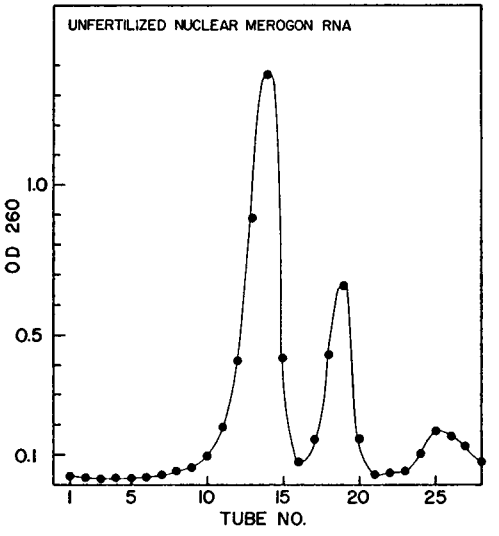


FIGURE 11 Sedimentation analysis of *Arbacia punctulata* unfertilized egg nuclear merogon total RNA. *Arbacia* unfertilized egg nuclear merogon total RNA was prepared by 4°C phenol extraction as described in Materials and Methods. Conditions of centrifugation and optical density assay are as indicated in Fig. 2.

analysis disclosed the variation in uridine and guanine characteristic of total RNA preparations containing 4S material. The base composition of the nucleated merogon was virtually identical with that of the intact unfertilized egg (Table II).

DISCUSSION

It should be noted that none of the embryonic material employed in this study was subjected to incubation under potentially anaerobic pulsing conditions, or to the massive dose of cold nucleic acid precursors commonly used as a chase. In addition, the above results are based solely on optical density and are therefore impervious to minor bacterial contamination.

The sedimentation profiles of *Arbacia* RNA as shown in Fig. 2 do not exhibit any of the obvious variations in the optical density profile observed as a function of

development in *Strongylocentrotus purpuratus* by Nemer (1963). In view of the agreement we have observed between profiles obtained from *Lytechinus variegatus* and *Lytechinus pictus* (Slater and Spiegelman, unpublished), and that of *Arbacia punctulata* RNA reported here, it is unlikely that the differences observed by Nemer are species dependent. Before any developmental significance can be ascribed to them evidence must be provided that they are not degradative artifacts of the techniques employed. In addition to this question of RNA integrity, complications due to bacterial contamination (Glisin and Glisin, 1964) and possible terminal additions in labeling experiments must also be considered (Gross et al., 1965).

The decline in the net yield of ribosomal RNA encountered subsequent to first cleavage may be related to degradation. A loss of cytoplasmic ribosomal RNA amounting to between 50 and 65% has been reported by Comb and Brown (1964) to take place during cleavage and blastulation. Since we observed no concomitant increase in 4S material or loss of resolution between major RNA components (which signifies breakdown) in parallel total RNA preparations, the aforementioned interpretation would necessitate that the ribosomal RNA was reduced to a non-ethanol-precipitable state in a highly coordinated fashion.

Direct comparisons relating the base compositions of high molecular weight RNAs prepared from *Arbacia punctulata* via phenol extraction to those of *Paracentrotus lividus* obtained by total TCA extraction (Elson et al., 1954) are of questionable validity (Spirin, 1960). However, the over-all results are in agreement with our own findings, suggesting that the metabolic modulations which accompany development do not appear to result in any gross changes in the general composition of the RNA.

The observed physical and chemical parity of RNA derived from nuclear merogons and intact eggs implies that no discernible qualitative polarity of high molecular weight RNA exists in the unfertilized egg. This conclusion concurs with the more decisive findings of Tyler (1965) that parthenogenically stimulated anuclear merogons initially exhibit a pattern of amino acid incorporation which is both qualitatively and quantitatively similar to that of the intact egg.

Hultin's (1961) contention that the absence of protein synthesis in mature unfertilized eggs resulted from some structural alteration affecting ribosomal competence has received new experimental support. Monroy et al. (1965) have presented evidence which leads them to conclude that the observed metabolic dormancy of these ribosomes can best be attributed to the presence of an inactivating protein coat and thus, by inference, not to any modification of the ribosomal RNA. In view of the striking physical similarity in ribosomal RNAs derived from latent ribosomes and their metabolically active counterparts as well as their agreement in chemical composition, our results conditionally support this conclusion insofar as the metabolic transitions prerequisite to ribosomal activation do not appear to require any gross alteration in their RNA.

It would appear, then, that the early events of embryogenesis do not alter the major physical or chemical characteristics of the bulk RNA native to the unfertilized egg. This overt consistency finds a parallel in the fact that few, if any, qualitatively new proteins are detectable prior to gastrulation (Pfohl and Monroy, 1962). Although the only species of RNA synthesized during this period is allegedly of an informational nature (Comb, 1965), its presence can not be detected without recourse to labeled precursors (Spirin and Nemer, 1965; Nemer and Infante, 1965). However, the protein synthesizing capabilities of "chemically enucleated" eggs (Gross and Cousineau, 1963) and nonnucleated egg fragments (Denny and Tyler, 1964) as well as some preliminary in vitro data (Maggio et al., 1964) suggest that the unfertilized egg may itself possess the template RNA prerequisite to the initial establishment of embryonic competence. It is evident that the next stage of the investigation must subject this suggestion to a rigorous test by a reliable quantitative assay for template RNA in the unfertilized egg. Direct evidence will be forthcoming that the unfertilized egg does in fact contain a substantial fraction of RNA, which, by virtue of its ability to direct amino acid incorporation in a defined in vitro system, can be conclusively regarded as translatable RNA (Slater and Spiegelman, in preparation).

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